Cell Surface Orifices of Caveolae and Localization of Caveolin to the Necks of Caveolae in Adipocytes^V

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Caveolae are noncoated invaginations of the plasma membrane that form in the presence of the protein caveolin. Caveolae are found in most cells, but are especially abundant in adipocytes. By high-resolution electron microscopy of plasma membrane sheets the detailed structure of individual caveolae of primary rat adipocytes was examined. Caveolin-1 and -2 binding was restricted to the membrane proximal region, such as the ducts or necks attaching the caveolar bulb to the membrane. This was confirmed by transfection with myc-tagged caveolin-1 and -2. Essentially the same results were obtained with human fibroblasts. Hence caveolin does not form the caveolar bulb in these cells, but rather the neck and may thus act to retain the caveolar constituents, indicating how caveolin participates in the formation of caveolae. Caveolae, randomly distributed over the plasma membrane, were very heterogeneous, varying in size between 25 and 150 nm. There was about one million caveolae in an adipocyte, which increased the surface area of the plasma membrane by 50%. Half of the caveolae, those larger than 50 nm, had access to the outside of the cell via ducts and 20-nm orifices at the cell surface. The rest of the caveolae, those smaller than 50 nm, were not open to the cell exterior. Cholesterol depletion destroyed both caveolae and the cell surface orifices.

INTRODUCTION

Caveolae were originally discovered by electron microscopy as noncoated invaginations of the plasma membrane of endothelial cells (Palade, 1953; Yamada, 1955). Similarly to so-called rafts, they are enriched in the membrane lipids cholesterol (Rothberg *et al.*, 1992; Schnitzer *et al.*, 1994; Gustavsson *et al.*, 1999; Pike *et al.*, 2002) and sphingomyelin (Liu *et al.*, 1997; Pike *et al.*, 2002) and perhaps also glycosphingolipids (Schnitzer *et al.*, 1995), phosphoinositides (Hope and Pike, 1996), and glycosyl phosphatidylinositol lipids (Parpal *et al.*, 1995). Noninvaginated plasma membrane rafts of these lipids may be precursors of caveolae, which become invaginated in the presence of the protein caveolin. Involvement of caveolae in a variety of cellular activities has been demonstrated, such as transcytosis, potocytosis (Anderson *et al.*, 1992), and uptake of cholesterol (Simons and Ikonen, 2000) and virus (Pelkmans *et al.*, 2001). Much attention has been on caveolae and rafts as foci and organizing centers for signal transduction (Shaul and Anderson, 1998; Smart *et al.*, 1999; Simons and Toomre, 2000; Marx, 2001). In particular, a regulatory role for caveolae in cell growth and differentiation has attracted interest (Smart *et al.*, 1999). Perturbation of caveolae/rafts may cause a wide range of disorders (Stahlhut *et al.*, 2000)—such as cancer

(Smart *et al.*, 1999), muscular dystrophy (McNally *et al.*, 1998), and Alzheimer's disease (Ikezu *et al.*, 1998; Simons *et al.*, 1998). Adipose cells are particularly rich in caveolae. In adipocytes the insulin receptor is localized and signaling in caveolae (Gustavsson *et al.*, 1999). Moreover, insulin-stimulated glucose uptake takes place in caveolae (Gustavsson *et al.*, 1996; Karlsson *et al.*, 2002).

Caveolae are usually seen as 50–100-nm omega- or flask-shaped invaginations of the plasma membrane by electron microscopy of cell thin-sections (Palade, 1953; Yamada, 1955; Rothberg *et al.*, 1992). Examination of critical point dried plasma membrane sheets covered with a thin-layer of chromium (Peters *et al.*, 1985), carbon-platinum deep-etch replicas of plasma membrane sheets (Rothberg *et al.*, 1992), and freeze-fractured plasma membranes (Westermann *et al.*, 1999) have demonstrated a roundish structure of caveolae with a narrowing at their attachment to the membrane. Analogously to clathrin, caveolin is believed to encase and cover the surface of the caveolae by means of its ability to polymerize (Rothberg *et al.*, 1992; Monier *et al.*, 1995). A striated coat seen on replicas after deep-etching has been interpreted as a clathrin-like caveolin cage encasing the caveolae (Rothberg *et al.*, 1992; Anderson, 1993). The existence of deep and shallow caveolae, apparently with specificity for caveolin-1 isoforms α and β , was described in human fibroblasts (Fujimoto *et al.*, 2000). However, only little detailed analyses of morphology of individual caveolae, or of caveolin localization in the caveola, or of caveolae distribution over the plasma membrane, have been reported. Thin-section electron microscopy on adipocytes has shown that caveolae are abundant and appear to be similarly shaped as in other cell types (Sheldon *et al.*, 1962; Williamson, 1964; Cushman, 1970; Jarett and Smith, 1974; Carpentier

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Abbreviations used: SEM, scanning electron microscopy; TEM, transmission electron microscopy.

et al., 1977a; Chlapowski *et al.*, 1983; Smith and Jarett, 1983). Images of perpendicular sections were complemented by sections oblique to the membrane (Chlapowski *et al.*, 1983) or sodium silicotungstate negatively stained membrane sheets (Vinten *et al.*, 2001). We have earlier prepared plasma membrane from 3T3-L1 adipocytes for electron microscopic examination by covering the inner surface of the plasma membrane with a thin layer of tungsten (Gustavsson *et al.*, 1999; Parpal *et al.*, 2001; Karlsson *et al.*, 2002). We have now applied this technique on intact adipocytes and plasma membranes of freshly isolated rat adipocytes. We give a high-resolution picture of the detailed structure of individual caveolae, demonstrating the localization of caveolin in the necks and not in the bulbs of caveolae. We also show the cell surface orifices of large caveolae and that a population of small caveolae have no outlet on the cell surface.

MATERIALS AND METHODS

Reagents

Rabbit anti-caveolin-1 polyclonal (C13630) and monoclonal (C43420) antibodies, and anti-caveolin-2 monoclonal (C57820) antibodies were from Transduction Laboratories (Lexington, KY). Rabbit polyclonal antimyc (SC789) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Colloidal gold conjugated anti-IgG was from Aurion (Wageningen, The Netherlands). Other chemicals were from Sigma-Aldrich (St. Louis, MO) or as indicated in the text.

Isolation and Incubation of Adipocytes

Adipocytes were isolated by collagenase digestion from Harlan Sprague Dawley rats (130–160 g, B&K Universal, Sollentuna, Sweden; Strålfors and Honnor, 1989). Cells were incubated in Krebs-Ringer solution (0.12 M NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄) containing 20 mM HEPES, pH 7.40, 1% (wt/vol) fatty acid-free bovine serum albumin, 100 nM phenylisopropyladenosine, 0.5 U·ml⁻¹ adenosine deaminase with 2 mM glucose, at 37°C on a shaking water bath.

Transfection of Adipocytes With Myc-Tagged Caveolin-1 and 2

Isolated adipocytes were transfected as described (Quon *et al.*, 1993; Nyström *et al.*, 1999) with modifications. Briefly, 200 μ l cells (40 μ l cell volume per ml) was mixed with 200 μ l of buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄, pH 7.5) containing 5 μ g of empty vector pcis2, pcis2 caveolin-1, or pcis2 caveolin-2 (supplied by Dr M. Quon, NIH, Bethesda, MD) in an electroporation cuvette. Cells were electroporated with six pulses at 600 V and 25 μ F using Gene pulser II (Bio-Rad, Hercules, CA). Cells from 15 cuvettes were pooled and kept at 37°C in 10% CO₂. After 1 h an equal volume of DMEM, pH 7.5, containing 25 mM glucose, 50 UI/ml penicillin, 50 μ g/ml streptomycin, 200 nM PIA, 7% (wt/vol) bovine serum albumin, and 25 mM HEPES, was added. After 18 h of incubation cells were collected, and plasma membranes were prepared for electron microscopy.

Culture and Transfection of Human Fibroblasts

Human foreskin fibroblasts AG-01518 were grown on gold-grids in DMEM supplemented with 50 UI/ml penicillin, 50 μ g/ml streptomycin, and 10% (vol/vol) newborn calf serum in 10% CO₂. For transfection, fibroblasts were transferred to medium without antibiotics and the following day were transfected with 1 μ g pcis2 caveolin-1 and Lipofectamine 2000 (Invitrogen) in 600 μ l for 5 h, according to the provided protocol (Invitrogen, Carlsbad, CA). The cells were then incubated for a further 24 h in medium without antibiotics.

Electron Microscopy of Plasma Membranes

After rinsing adipocytes in ice-cold phosphate buffer (10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 137 mM NaCl), they were attached to gold grids (Voldstedlund *et al.*, 1993): Poly-L-lysine-formvar-coated grids were rehydrated in ice-cold phosphate buffer containing the cells. Grids with captured adipocytes were flushed with ice-cold 150 mM KCl, 1.9 mM Tris-HCl buffer, pH 7.4.

Human foreskin fibroblasts were washed as above and then briefly (90 s) treated with hypotonic buffer (50 mM HEPES, pH 7.5) followed by 30 s in poly-L-lysine (1 mg/ml in 150 mM HEPES, pH 7.5), before probe-sonication for 5 s (Robinson *et al.*, 1992).

Plasma membranes remaining on the grids were washed three times in 150 mM HEPES, pH 7.5, and fixed in 0.1 M sodium cacodylate, containing 0.1 M

sucrose, 3% paraformaldehyde, and 0.05% glutaraldehyde, for 30 min at room temperature.

Membranes were blocked for 60 min at 37°C with 1% bovine serum albumin (BSA-c, Aurion), 0.1% gelatin, and 1% normal goat serum (Aurion), followed by anticaveolin antibodies for 2 h at 37°C. Grids were rinsed in phosphate buffer, with 0.15% BSA-c, pH 7.5, before incubation with secondary antibodies. Goat anti-rabbit or anti-mouse IgG, conjugated with 6 or 15 nm colloidal gold, was added to plasma membranes and incubated overnight at 4°C.

After immunolabeling, plasma membranes were rinsed and fixed in 2% glutaraldehyde for 10 min followed by 1% OsO₄ for 30 min in 0.1 M sodium cacodylate, with 0.1 M sucrose, pH 7.5, at room temperature. Grids were rinsed with water, frozen, lyophilized, and coated with 2 nm tungsten by magnetron sputtering directly in the freeze-dryer (Lindroth *et al.*, 1991). Transmission electron microscopy (TEM) was with Jeol EX1200 TEM-SCAN (Tokyo, Japan). Scanning electron microscopy (SEM) was with LEO 1550 Gemini. No labeling was observed neither in the absence of the primary antibody nor cross-reactivity between secondary and primary antibodies.

Electron Microscopy of Intact Cells

Adipocytes were rinsed in ice-cold phosphate buffer and fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate for 1 h at room temperature. Cells were attached to grids as above. Cells were then further fixed with 1% OsO₄ in 0.1 M sodium cacodylate, containing 0.1 M sucrose, pH 7.5, for 2 h at room temperature. Grids were rinsed with water, frozen, lyophilized, and coated with 1 nm tungsten by magnetron sputtering directly in the freeze-dryer (Lindroth *et al.*, 1991). SEM was with LEO 1550 Gemini (Zeiss, Oberkochen, Germany).

Immunofluorescence Deconvolution Microscopy

Adipocytes were rinsed in phosphate buffer and prefixed in 0.1 M sodium cacodylate containing 3% paraformaldehyde and 0.05% glutaraldehyde for 30 min at room temperature. After treatment with 0.1% NaBH₄ for 15 min unspecific binding was blocked with 1% BSA-c, 0.1% saponin, 0.1% gelatin, 1% normal goat serum (Aurion) for 1 h at 37°C. Cells were then incubated with primary antibodies (rabbit anticaveolin, 40 μ g/ml) in 0.1% saponin for 1.5 h at 37°C. Fluorescent secondary antibody (Alexa fluor 488) was detected by fluorescence microscopy (Axiovert 200M; Zeiss). Image stacks with number of planes as indicated were collected using Axiovision 3.1 (Zeiss). Images were deconvoluted using Maximum Likelihood Estimation algorithm by Huygens v2.3.1a-64 software (Scientific Volume Imaging, Hilversum, The Netherlands). 3D rendering was by Imaris 3.1.3 software (Bitplane AG, Zurich, Switzerland). Labeling was not observed in the absence of the primary antibody or in the cross-reactivity between secondary and primary antibodies.

RESULTS

Caveolae Openings on the Cell Surface

Examination by SEM of the outer surface of freshly isolated adipocytes revealed that the cell surface was dotted with numerous pores of similar size (Figure 1).

The diameter of the orifices was ~20 nm (Figure 1C). Closer view into the actual openings was not possible because the large adipocytes were not completely static; thus micromovements caused blurring of images at higher magnification. The orifices were nonrandomly distributed over the cell surface with patches of fewer orifices per surface area (Figure 1C). In the typical, denser regions there were 24 ± 1.2 orifices/ μ m² (mean $\pm$ SEM, $n = 5$ cells) and on average over the whole adipocyte surface there were 20.7 ± 2.4 openings/ μ m² (mean $\pm$ SEM, $n = 75$ areas; counting 15 areas on 5 separate cells covering 60 μ m² per cell), which corresponds to $\sim 6 \times 10^5$ orifices in a cell sized 100 μ m in diameter.

Caveolin Localization in the Individual Caveolae

As shown in Figure 2 we found caveolae of greatly varying sizes to protrude from the inner surface of the plasma membrane. The smallest caveolae, consistently labeled for caveolin and thus identified as caveolae, were ~25 nm in diameter, whereas the largest were ~150 nm. Close-ups of individual caveolae showed a balloon-like shape with narrow necks or stems connecting them to the plasma mem-

Figure 1. SEM of adipocyte surface. Freshly isolated adipocytes were fixed, attached to grids, cryosputtered and examined by SEM.

brane proper (Figure 3). Caveolin-1 and -2 labeling was restricted to these necks (Figure 3), using both polyclonal and monoclonal antibodies. To further test this finding we transfected adipocytes with caveolin-1 or -2 that were C-terminally myc-tagged. Using antimyc antibodies, also these caveolins were detected only in the necks and not in the bulbs of the caveolae (Figure 4). However, we did not detect any necks for caveolae smaller than ~50 nm diameter, but caveolin labeling nonetheless appeared restricted to one part of the caveola (see Figure 7C). We found some caveolae that were attached by two necks (Figure 3D). A further peculiarity sometimes encountered was the apparent joining of two

Figure 2. Electron micrographs of the inside of adipocyte plasma membranes. Plasma membrane sheets of freshly isolated adipocytes were prepared attached to grids, cryosputtered and examined by TEM (A) or SEM (B). Images have been contrast inverted.

caveolae by a neck (Figure 3E), a phenomenon previously indicated in micrographs of endothelial cells (Uehara and Miyoshi, 1999).

The most common caveolae "cluster" consisted of one caveola with protrusions, like a budding caveola (Figure 2B). Occasionally small clusters with 5–6 caveolae were seen, but not the large clusters of the grape-cluster type described earlier for other cells.

Spiral or bipolar striations have been observed on the surface of caveolae (Peters *et al.*, 1985; Rothberg *et al.*, 1992) and have been suggested to consist of oligomeric caveolin filaments in human skin fibroblasts (Rothberg *et al.*, 1992; Fernandez *et al.*, 2002). We therefore examined human fibroblasts by the same procedure that we used for adipocytes. Caveolae were frequent in the plasma membrane of the fibroblasts, but we did not detect distinct striations or

Figure 3. TEM close-ups of individual caveolae labeled against caveolin. Plasma membrane sheets from freshly isolated adipocytes were attached to grids and incubated with antibodies against caveolin for immunogold labeling. Membranes were then cryo-sputtered and examined by TEM. Images have been contrast inverted. Scale bar, 100 nm.

decorations of the caveolar surfaces (Figure 5), to the extent previously described (Peters *et al.*, 1985; Rothberg *et al.*, 1992). Clathrin-coated pits, which were quite frequent in the fibroblasts, displayed the expected honeycomb surface pattern (Figure 5), and filaments in the plane of the membrane were common (Figure 5). Similarly to the findings with adipocytes, we found caveolin to be

strongly concentrated at the membrane proximal part of the caveolae in the fibroblasts (Figure 6A). To further test this finding, we transfected the fibroblasts with myc-caveolin-1 and analyzed for immunogold labeling of the myc-tag. Also myc-caveolin-1 labeling was with few exceptions found at the membrane proximal region of the caveolae (Figure 6B).

Figure 4. TEM of caveolae immunogold labeled for myc-tagged caveolin. Freshly isolated adipocytes were transfected with C-terminally myc-tagged caveolin-1 (A) or -2 (B). Plasma membrane sheets attached to grids were prepared and immunogold-labeled against the myc-tag, cryosputtered, and examined by TEM. Images have been contrast inverted.

Ruthenium red stains the adipocyte plasma membrane without passing through it. Treatment of the intact adipocyte with ruthenium red stained virtually all caveolae with a diameter larger than ~ 50 nm, but rarely smaller caveolae (Figure 7A), although these contained caveolin (Figure 7C). The bulbs as well as necks of the larger caveolae were stained with ruthenium red (Figure 7B). The necks are thus hollow and continuous with the plasma membrane and bulbs of caveolae.

It has earlier been shown in other cell types that cholesterol depletion of cell membranes leads to the loss of caveolae structures (Chang *et al.*, 1992; Schnitzer *et al.*, 1994; Gustavsson *et al.*, 1999). When we thus treated the adipocytes with β -cyclodextrin to deplete the plasma membrane of $\sim 50\%$ of its cholesterol, caveolae disappeared from the inner face of the plasma membrane (Figure 8A). The caveolin protein stayed clustered in the cholesterol and caveolae-depleted plasma membrane (Figure 8A). The orifices on the cell surface were also no longer visible after the cholesterol depletion (Figure 8B), suggesting that the orifices represent the caveolar openings to the outside of the cell.

Figure 5. TEM of plasma membrane from human fibroblasts. Plasma membrane sheets of the fibroblasts were prepared attached to grids, cryosputtered, and examined by TEM. Image has been contrast inverted.

Caveolae With and Without Access to the Outside of the Cell

The density of caveolae varied greatly over the adipocyte plasma membrane, but most of the plasma membrane was rich in caveolae as seen from a large number of EM pictures (e.g., Figure 2B with a caveolae-dense region bordering to a low-density patch to the right; cf. Peters *et al.*, 1985). Patches of lower caveolin labeling density were scattered over the cell membrane as seen from a more global perspective of caveolin-labeling and immunofluorescence light microscopy (Figure 9).

The definitions of caveolae-dense and low-density regions are obviously arbitrary and any randomly chosen area can contain contributions from both. To estimate the caveolae density and size distribution, we have therefore counted and measured the caveolae in what we see as clearly caveolae-dense (Figure 10A) or low-density regions (Figure 10B), respectively, thus avoiding the problem with transitional areas. The total number of caveolae (25–150 nm diameter) was ~ 43 caveolae/ μm^2 in the caveolae-dense regions of the cell and ~ 16 caveolae/ μm^2 in caveolae low-density patches. In a 100- μm adipocyte this corresponds to a total of $\sim 10^6$ caveolae/cell. In the caveolae-dense regions the number of caveolae with a diameter >50 nm, i.e., those that are connected with the cell surface, was 22 per μm^2 (Figure 10A). A similar number was obtained by counting the density of ruthenium red-stained caveolae. These figures, in turn, compare very well with the density of openings on the surface of the cell (above).

About 50% of the caveolae were <50 nm, caveolae that appeared not to be open to the outside of the cell. The low-density patches of caveolae were especially lacking in caveolae with a diameter >50 nm. However, although the number of caveolae larger than 50 nm accounted for 50% of caveolae, the surface area of these large caveolae accounted for 80% of the total caveolae membrane area. The total sum of caveolae (25–150 nm diameter) membrane increased the plasma membrane area by 50%.

DISCUSSION

The most striking morphological feature of the adipocyte plasma membrane is the abundance of caveolae orifices on the outer surface and the corresponding caveolae bulbs covering much of the inner surface. Although known to exist, the caveolae orifices on the outside of the cell have to our knowledge never been demonstrated before. Because both

Figure 6. TEM close-ups of caveolae labeled against caveolin in human fibroblasts. (A) Plasma membrane sheets of the fibroblasts were prepared attached to grids and incubated with antibodies against caveolin for immunogold labeling (6 nm gold). Membranes were then cryosputtered and examined by TEM. (B) Fibroblasts were transfected with C-terminally myc-tagged caveolin-1. Plasma membrane sheets attached to grids were prepared and immunogold-labeled against the myc-tag (6 nm gold), cryosputtered, and examined by TEM. Scale bar, 100 nm.

the bulbs and necks of the caveolae were accessible to extracellular ruthenium red, it appears that the neck is the duct that connects the caveola bulb with the orifice on the outside of the cell. These conclusions are supported by the similar distribution and density of caveolae of >50 nm in diameter, of ruthenium red-stained caveolae, and of the orifices on the cell surface. The parallel effects of cholesterol depletion on cell surface orifices and on caveolae structures suggest that the cell surface orifices indeed represent the caveolar outlets.

Figure 7. TEM of plasma membranes from ruthenium red-stained adipocytes. Freshly isolated adipocytes were incubated with 1 mg/ml ruthenium red during the glutaraldehyde/paraformaldehyde prefixation. Plasma membrane sheets attached to grids were immunogold labeled against caveolin, treated as described in MATERIALS AND METHODS and examined by TEM. (A) Overview of the inner surface of the plasma membrane. Black arrowhead, ruthenium red-stained caveolae; white arrowhead, nonstained caveolae. (B) Close up of stained caveola. (C) Close up of stained and small nonstained caveolae.

A striated spiral coat of caveolae, suggested to be formed by caveolin, has been reported for carbon-platinum replicas of cultured human fibroblasts after deep-etching (Rothberg *et al.*, 1992). Similar striped, but bipolar, surface structures were described on caveolae of capillary endothelial cells after critical point drying and coating with chromium (Peters *et al.*, 1985). We did not see a striated coat in the adipocytes, neither by TEM nor SEM. In this study membranes were lyophilized and coated with a very thin (1–2 nm) layer of tungsten (Lindroth *et al.*, 1991), which produces an extremely homogeneous layer of metal and which is not prone to decorating artifacts (Lindroth and Sundgren, 1989). We have, furthermore, used cryosputtering to reduce artifacts due to rehydration or contamination (Lindroth *et al.*, 1991). We therefore believe that adipocyte caveolae do not exhibit striated coats. Moreover, anticaveolin-1 or -2 labeling of caveolae with identifiable necks was distinctly restricted to the necks that attach the caveolae to the membrane, with

Figure 8. EM of adipocyte surface and inside of plasma membrane after cholesterol depletion. Freshly isolated adipocytes were depleted of ~50% of the plasma membrane cholesterol by incubation with β -cyclodextrin at 10 mM for 50 min. (A) Plasma membrane sheets were prepared attached to grids. After immunogold labeling against caveolin membranes were examined by TEM. Image has been contrast inverted. (B) Intact cells were fixed, attached to grids, cryosputtered, and examined by SEM.

very limited labeling of the caveolar bulbs. This is unexpected because the general belief is that caveolin encases the caveola bulb by virtue of its ability to form homo-oligomeric complexes between caveolin-1 and hetero-oligomeric complexes between caveolin-1 and -2 (Rothberg *et al.*, 1992; Monier *et al.*, 1995; Marx, 2001; Parton, 2001; Fernandez *et al.*, 2002). Different antibodies toward caveolin-1 and -2 gave the same result. Moreover, myc-tagged caveolin-1 or -2 was expressed only in the necks of the caveolae, as determined with antibodies against the myc-tags. Because both the N- and C-termini of caveolin are cytoplasmic, it is unlikely that a myc-tag at the C-terminal would become inaccessible in the bulb of the caveola. It is possible that caveolin present in the bulb is washed off the caveolae during preparation of the membranes for immunolabeling; however, in such case caveolin binding to the bulbs of caveolae would be qualitatively different from that of caveolin binding in the necks. Taken together, our findings demonstrate that in adipocytes caveolin proteins are present in the necks of caveolae and do not form a stable cage around the caveola bulb. It is interesting that isolated caveolae examined by freeze-fracture

Figure 9. Caveolin distribution over the adipocyte. Freshly isolated adipocytes were fixed and immunofluorescence labeled with antibodies against caveolin in the presence of saponin to permeabilize the cells. Cells were then examined by immunofluorescence microscopy in stacks of 150-nm focal plane shifts, 200 images were deconvoluted, and a 3D image was rendered. Shown is a transverse section of the cell, seen from above, the nucleus making a protrusion at the top right of the image.

electron microscopy exhibited no striation, and caveolin was only found at a single spot on each caveola, close to what appeared to have been the caveolae openings (Westermann *et al.*, 1999). Our findings that caveolae in human fibroblasts did not present clear striations when examined by TEM and, importantly, that caveolin labeling was restricted to the membrane proximal part of the caveolae suggest that this may not be unique to adipocytes. However, different levels of expression of caveolin-1 and -2 and caveolae in different cell types may explain some of the reported differences regarding caveolae structure and function.

Caveolin is known to be critical for formation of caveolae, as shown by induction of caveolin expression in cells not normally expressing the protein (Fra *et al.*, 1995) and by the absence of caveolae in cells from caveolin-1 null mice (Drab *et al.*, 2001; Razani *et al.*, 2001). Our findings indicate that caveolin presence in the bulb is not necessary to maintain the bulb. It therefore appears that caveolin functions not to directly keep the shape of the caveolae, but to sequester the caveolar membrane and its lipids from the rest of the membrane, not unlike tight junctions, and thus indirectly form caveolae. This suggests that caveolae form from rafts by membrane expansion inside a caveolin "membrane lock" through accretion of its lipid components. The localization of caveolin in the necks has implications for an understanding of the reported interaction, including coprecipitation, of caveolin with a large number of different proteins. This could mean that caveolin protein interaction, similarly as discussed for the lipids, is important for recruiting proteins to caveolae and that much of the *in situ* interaction is transient. This does not contradict previous observations because immunoprecipitation can only demonstrate a propen-

Figure 10. Distribution in the plasma membrane of caveolae of different diameters. Plasma membrane sheets were prepared and immunogold-labeled against caveolin for examination by TEM. The size and number of caveolae were determined manually from prints. (A) Typical caveolae-dense regions; 1552 caveolae in five cells from three different preparations, covering a total membrane area of $36 \mu\text{m}^2$ were measured. (B) Caveolae low-density patches, 630 caveolae in five cells from three different preparations, covering a total membrane area of $36 \mu\text{m}^2$ were measured. Mean $\pm$ SEM.

sity for interaction, not that an interaction takes place in the intact cell. It will be interesting to examine the localization in adipocytes of dynamin in relation to caveolin, because dynamin was found in caveolae necks of lung endothelium (Oh *et al.*, 1998).

By TEM and immunogold labeling against caveolin, we identified caveolae that varied in size from 25 to 150 nm. It is interesting that only half of these, those larger than ~ 50 nm, were open to the outside of the cell, as determined by accessibility for ruthenium red. The number of large (>50 nm) stained bulbs matches the number of openings on the outside of the cell. They were concentrated in the large caveolae-dense regions, and very few of them were found in the caveolae low-density patches, a pattern mirrored by orifices on the surface. The small <50 nm membrane attached caveolae vesicles were labeled by anticaveolin antibodies and destroyed by cholesterol depletion, which strongly indicates an intimate relation with the large caveolae. They may be vesicles about to fuse with or to bud off from the membrane. Potocytosis has been suggested to involve transiently formed and sealed off compartments that are independent from the extracellular space, but still contiguous with the plasma membrane (Anderson *et al.*, 1992). Our finding that the sealed-off caveolae are invariably very small may indicate a more specialized function. It will obviously be important to find methods to isolate these separately from the larger caveolae in order to characterize them biochemically.

Larger structures containing caveolae have been described in 3T3-L1 adipocytes (Gustavsson *et al.*, 1999; Baumann *et al.*, 2000) and was recently shown to contain all elements of the plasma membrane (Parton *et al.*, 2002). It is noteworthy that these structures were not found in the primary fat cells used in this study and emphasizes the importance of using electron microscopy when studying caveolae as well as using physiologically relevant cells.

The number of caveolae determined here is on par with previous estimates of 45 intramembraneous invaginations per μm^2 (Carpentier *et al.*, 1977b) or 30 plasma membrane-associated microvesicles per μm^2 (Chlapowski *et al.*, 1983). They are higher than the estimated number of caveolae in 3T3-L1 adipocytes: 10 per μm^2 (Fan *et al.*, 1983). This is of interest because for instance the insulin receptor, which is located in caveolae (Gustavsson *et al.*, 1999), numbers $\sim 2 \times 10^5$ receptors/adipocyte (Gammeltoft, 1988) and hence will be found in a subset of the 10^6 caveolae even if evenly distributed.

The large number of caveolae increases the plasma membrane surface area considerably and, consequently, a substantial fraction of the plasma membrane constitutes caveolae membrane. From the caveolae diameters and number of caveolae we calculate that one third of the plasma membrane surface area is made up of caveolae membrane, which is similar to previous estimates from electron microscopy of adipocyte thin sections (Chlapowski *et al.*, 1983). The large adipocyte (50–150- μm diameter) has a very unfavorable surface area to cell volume ratio combined with a massive flux of fatty acids over the plasma membrane. During maximal lipolysis (0.7 μmol fatty acid per min per ml packed cells; Belfrage *et al.*, 1984) 10^4 – 10^5 molecules of fatty acids/ $\text{s}/\mu\text{m}^2$ pass over the plasma membrane. Free fatty acids in the plasma membrane cause prompt lysis of the cells (Strålfors, 1990). Caveolin-1 and -2, which have been described to bind fatty acids (Trigatti *et al.*, 1999), may facilitate the flow of fatty acids over the plasma membrane via the caveolae membrane expansion. Indeed, caveolin-1-deficient mice

have a corrupt adipose tissue and elevated serum concentration of free fatty acids, especially in the postprandial state (Razani *et al.*, 2002). This can be attributed to the fatty acid binding and transport function of caveolin-1/caveolae and to increased fatty acid release resulting from insulin resistance, as we have previously shown in adipocytes lacking caveolae due to cholesterol depletion (Parpal *et al.*, 2001). Moreover, the fatty acids have to pass to and from the adipocyte and the blood stream via endothelial cells, which are second only to adipocytes in terms of number of caveolae and amount of caveolin. Considering that for most of the plasma membrane surface the corresponding cytosol of an adipocyte is no more than 200–400 nm thick, elaborate caveolar clusters may not be needed or cannot be allowed for lack of space. Caveolae clusters are, however, common in, e.g., 3T3-L1 adipocytes (Novikoff *et al.*, 1980; Gustavsson *et al.*, 1999) and is a prominent phenomenon in endothelial cells (Uehara and Miyoshi, 1999). They are also described in severely starved and fat depleted rat adipocytes (Sheldon *et al.*, 1962; Williamson, 1964), perhaps a response adaptation to a massive and prolonged efflux of fatty acids.

In conclusion, using SEM we have shown cell surface orifices that represent caveolae openings. We have demonstrated that in adipocytes there are two populations of caveolae, one larger than 50 nm open to the cell surface and one smaller than 50 nm with no cell surface openings. Caveolin localization was restricted to the caveolae necks, connecting the caveola to the plasma membrane. In the small caveolae caveolin also appeared polarized, although no necks were found. Moreover, also in human fibroblasts caveolin labeling was restricted to the membrane proximal parts of caveolae.

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